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Seventh day of January 2004

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AUSTRALIA

Patents Act 1990

The University of Melbourne

PROVISIONAL SPECIFICATION

Invention Title:

Size Exclusion Electrophoresis

The invention is described in the following statement:

Technical Field

The present invention related to size exclusion electrophoresis using hydrogels.

Background Art

There is a constant need for improved separation technologies for the purification of proteins and other biological macromolecules of significance to analytical and preparative biotechnological applications. Hydrogels, in various forms, constitute an important matrix for many biotechnological separation technologies, which include electrophoresis gels, electrophoresis membranes and chromatography media. Most of the polymers used for the manufacture of these hydrogels are either based on polysaccharides or polyacrylamide, both of which have certain limitations. The polysaccharides used are normally based on naturally occurring raw materials which often have associated chemical and structural impurities. Polyacrylamide, while a very widely used polymer with many good properties, is based on a neurotoxic monomer and is furthermore susceptible to hydrolysis at alkaline pH. There is therefore clearly a need to develop new hydrogels based on pure, stable and less toxic monomers. Poly hydroxyethyl methacrylate (poly HEMA) has been shown to be a good material for the manufacture of polymeric networks and has been approved by the US Food and Drug Authority in a number of products used for medical applications. The monomer HEMA is far less toxic than acrylamide, but the limited solubility of HEMA and poly HEMA in water has so far limited their use in hydrogels.

The aim of this work was to develop hydro-organic solvent systems suitable for the polymerization of monomers such as HEMA into new polymeric matrix structures that can be applied to the separation of biomacromolecules. In particular, new matrices were developed for electrophoretic separation of proteins. A new type of membrane system was also developed to be used in separating bio-macromolecules under electrophoretic conditions. New pathways to network formation of hydrogels in hydro-organic media were developed and new properties based on this network were evaluated. The consequence of the new network structures will give novel hydrogels that can be used to separate proteins under electrophoretic condition in the manner of size exclusion chromatography (SEC). Proteins with large molecular weights will be eluted out first during their separations, which contrasts with existing technologies where large molecules are separated last and are sometimes difficult to elute or separate. The new technology will be capable of separating, in particular large bio-molecules, such as

Fibrinogens, DNA and RNA, which are usually difficulty to separate effectively in high purity by existing methodologies.

The present inventors have developed a new hydrogel medium that allows the separation of compounds by size under the electrophoretic conditions.

Disclosure of Invention

In a first aspect, the present invention provides a method for separating one or more compounds according to size using electrophoresis, the method comprising:

(a) providing a medium in the form of polymeric hydrogel having a network containing macropores and micropores;

(b) adding one or more compounds to part of the medium; and

(c) applying an electric potential causing at least one compound to pass through the medium, wherein movement through the medium is related to the size of the compound.

In a second aspect, the present invention provides a size exclusion electrophoresis system comprising:

(a) a cathode;

(b) an anode; and

(c) a separation medium in the form of polymeric hydrogel having a network containing macropores and micropores capable of separating a mixture of compounds according to size, the medium disposed between the anode and cathode.

In a preferred form, the system further includes means for supplying a sample containing one or more compounds to be separated to the system.

In a preferred form, the system further includes means for retaining or capturing a compound separated by the system.

In a preferred form, the system further includes a voltage supply and means for applying an electric potential between the cathode and anode.

The system can be formed by having the separation medium disposed between two ion-permeable barriers forming two chambers either side of the size exclusion medium. Sample containing the compound(s) to be separated can be placed in one of the chambers and, under the influence of the applied voltage, a compound will move through the separation medium in accordance with its size (large molecules elute out first) to the second chamber where it can be retained or collected. It is also possible to

have a plurality of different separation media disposed between the cathode and anode. In this form, preferably each separation medium would have a different pore structure so as to be able to separate compounds of different size.

In a third aspect, the present invention provides use of a separation medium in the form of polymeric hydrogel having a network containing macropores and micropores in size exclusion electrophoresis.

Preferably, the polymeric hydrogel comprises a network of macropores and micropores formed by copolymerizing at least one monomer having at least one double bond and at least one crosslinker having at least two double bonds in the presence of a polymeric additive forming a hydro-organic system with water.

The monomer having at least one double bond may be selected from esters of acrylic and methacrylic acid, or polyol esters of acrylic or methacrylic acid. Typical polyols are polyethylene glycol, a range of polyethylene glycol esters or ethers, polypropylene glycol, a range of polypropylene glycol esters or ethers, random or block copolymers of ethylene glycol and propylene glycol, or any suitable polyols such as glycerol, pentaerythritol, ethylene glycol or propylene glycol which are fully or partly esterified. Mixtures consist of at least two of the above monomers can also be used.

Mixtures of the above monomer with any other well-known monomers suitable for free radical polymerization may be used.

Preferably use of above monomer with greater than 50% in the mixture of monomers; more preferably greater than 80%.

Preferably, the monomer is one or more hydrophilic monomers from the esters of acrylic acids.

In one preferred form, the monomer is hydroxyethyl methacrylate (HEMA).

The crosslinker having at least two double bond may be selected from esters of acrylic and methacrylic acid, or polyol esters of acrylic or methacrylic acid. Typical polyols are polyethylene glycol, a range of polyethylene glycol esters or ethers, polypropylene glycol, a range of polypropylene glycol esters or ethers, random or block copolymers of ethylene glycol and propylene glycol, or any suitable polyols such as glycerol, pentaerythritol, ethylene glycol or propylene glycol which are fully or partly esterified (for example, glycerol can be esterified with methacrylic acid to give the crosslinking mixture). Mixtures consist of at least two of the above crosslinkers can also be used.

Mixtures of above crosslinker with any other well-known crosslinkers suitable for free radical polymerization may be used.

Preferably use of the above crosslinker with greater than 50% in the mixture of crosslinkers; more preferably greater than 80%.

In one preferred form, the crosslinker is ethylene glycol dimethacrylate (EGDMA).

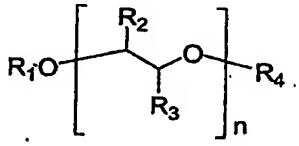
Preferably, the polymeric hydrogel is made from a mixture of monomer content of about 10 to 40%M and crosslinker of about 1 to 30%X before polymerization. When HEMA and EGDMA are used, the preferred compositions of monomer mixture of HEMA with EGDMA are less than about 40% M and less than about 20% X. It will be appreciated, however, that other concentrations can be used depending on the monomer and crosslinker used.

Any suitable free radical producing method can be used as the initiation system. The initiation system is preferably formed by the redox, thermal or photo initiator/s. More preferably, the redox initiator is formed by ammonium persulphate (APS) with *N,N,N',N'*-tetramethylethylenediamine (TEMED).

The polymeric addictive is preferably a hydrophilic polymer miscible with water and miscible with a linear polymer produced from the monomer used for copolymerization; or a hydrophilic polymer miscible with water and has a similar solubility parameter ($\pm 10(MPa)^{0.5}$) to that of a polymer produced from the monomer used for copolymerization. The polymeric additive can be single entity acting as both a porogen to form macropores during the polymerization and a solvent with water to form the hydro-organic solvent.

The polymeric additive is preferably selected from ethylene glycol or polyethylene glycol, propylene glycol or polypropylene glycol, random or block copolymers of any of the above mixtures, or any of the above additives that have an ester or ether end group. Mixtures consist of at least two of the additives can also be used.

More preferably, the polymeric additive has the following general formulation:



R_1, R_4 = H, CH_3, $-(CH_2)_X-CH_3$ (x=1-4),
$-C(=O)-R_5$ ($R_5=(CH_2)_X-CH_3$ (x=0-4))

R_2, R_3 = H, CH_3, $-(CH_2)_X-CH_3$ (x=1-4), OH

In a preferred from, the polymeric additive is a polyethylene glycol or polypropylene glycol. The polyethylene glycol preferably has a molecular weight range from about 100 to 100000; preferably from about 200 to 10000; more preferably from about 400 to 4000.

The polypropylene glycol typically has a molecular weight range from about 100 to 100000; preferably from 200 to 10000; more preferably from about 58 to 600.

In another preferred form, the polymeric additive is a copolymer with a hydrophilic component and a hydrophobic component. Preferably, the polymeric additive is a copolymer of polyethylene glycol with polypropylene glycol.

Due to the molecular arrangement of the medium, large molecules pass through the medium via the macropores relatively quickly as there is a shorter distance to travel. Smaller compounds, however, pass into or through the micropores and therefore travel a further distance and thus have a greater retention time in the medium.

As an electromotive force is applied via the voltage, different compounds can be caused to move through the medium according to their charge.

The medium can be in the form of a slab, capillary or a membrane arrangement. The medium can be used to replace other media which are presently used in size exclusion chromatography applications and apparatus. The method according to the present invention is particularly suitable for use in a membrane-based electrophoresis apparatus.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following drawings and examples.

Brief Description of the Drawings

Figure 1 is a schematic representation of **a)** ultra filtration membrane, **b)** novel membrane with large pores which are interconnected by smaller diffusive pores, and **c)** size exclusion column.

Figure 2 is a phase diagram for poly(HEMA).

Figure 3 is schematic diagram of the size exclusion action of a hydrogel suitable for size exclusion electrophoresis.

Figure 4 is a schematic diagram of a membrane-based electrophoresis separation cartridge which can house a size exclusion hydrogel for size exclusion electrophoresis.

Mode(s) for Carrying Out the Invention

DEFINITIONS

The following terms shall have the indicated definitions unless otherwise indicated:

"Hydrogel" is a chemically crosslinked polymer characterized by hydrophilicity and insolubility in water.

"Micropores" are pores within the gel network of the background matrix. The size of these pores can be related to the hydrogel formation species in the initial pre-gelling mixture using relationships and theories developed for common electrophoretic matrixes. For example, micropores within an acrylamide hydrogel are related to the total monomer concentration and monomer to crosslinker ratios in the free radical polymerization of acrylamide and N,N'-methylenebisacrylamide (Bansil, R.; Gupta, M. Ferroelectrics **1980**, 30, 64).

"Macropores" are pores within the membrane that are significantly larger (more than 2 times) than micropores of the background matrix.

"Microporous membrane" is a separation membrane having a substantially continuous interconnecting micropores. Such membranes are used extensively in preparative electrophoresis.

"Macroporous membrane" is a separation membrane having continuous interconnecting micropores but non-continuous macropores (i.e. macropores are not connected directly to each other). Such membranes have similar sieving properties to the corresponding microporous membrane, but allows for higher flow rate through the matrix because of the reduced diffusional constraints.

"Size exclusion membrane (SE-Mem)" is a bi, or multimodal separation membrane having continuous interconnecting micropores, and interconnecting macropores within its matrix. SE-Mem can have different separation behaviours depending upon the size of the micropores (S_{mic}), the size of the macropores (S_{mac}) and the size of the bio-molecule mixture (S_{bio}). When $S_{bio}>S_{mac}>S_{mic}$, no separation would occur; when $S_{mac}\sim S_{bio}>S_{mic}$, all molecules with dimension smaller than the macropores would be separated from their bigger counter part; when $S_{mac}>S_{bio}\sim S_{mic}$, all molecules with dimension smaller than the macropores would be separated from their bigger counter part, and be eluted in the order of decreasing size.

From the above description of SE-Mem, the challenge in producing such membrane lies in i) increase the size exclusion limit, i.e. the size of the largest interconnecting pores, and ii) produce a polymer with both interconnecting micropores and macropores. It would be a substantial advantage to develop a simple process to synthesis such membrane.

Multi-modal HEMA hydrogels are suitable to be used as SE-Mem as two general types of pores exist in such membrane - macropores formed by the template or porogen, and micropores formed by the crosslinking of polymer chains. The size exclusion limit of such membrane is also increased because of the macropores.

SE-Mem can be used in membrane based electrophoresis techniques and as membrane support for membrane chromatography and affinity membrane chromatography. It can take the form of flat sheet, stacked sheet, radical flow cartridges, hollow fibre molecules, slab, and column.

The term "stream 2 (S2)" is used in this specification to denote the second interstitial volume where material is moved from the first interstitial volume through the separation membrane to a stream of the electrophoresis apparatus. This stream may also be called the "downstream".

The term "stream 1 (S1)" is used in this specification to denote the first interstitial volume, which may also be called the "upstream".

The term "forward polarity" is used when the first electrode is the cathode and the second electrode is the anode in the electrophoresis apparatus and current is applied accordingly.

The term "reverse polarity" is used when polarity of the electrodes is reversed such that the first electrode becomes the anode and the second electrode becomes the cathode.

%M refers to the total concentration of monomer as a weight percentage; %X refers to the number of double bonds on the crosslinkers as a portion of the total number of double bonds on the monomers.

$$\%M = \frac{\text{total mass of monomers (g)}}{\text{mass of reaction mixture (g)}} \times 100$$

$$\%X = \frac{\text{number of double bonds on crosslinkers (mol)}}{\text{total number of double bonds on monomers (mol)}} \times 100$$

Membrane-Based Electrophoresis

A number of membrane-based electrophoresis apparatus developed by Gradipore Limited, Australia were used in the following experiments. In summary, the apparatus typically included a cartridge which housed a number of membranes forming two chambers, cathode and anode connected to a suitable power supply, reservoirs for samples, buffers and electrolytes, pumps for passing samples, buffers and electrolytes, and cooling means to maintain samples, buffers and electrolytes at a required temperature during electrophoresis.

The cartridge contained three substantially planar membranes positioned and spaced relative to each other to form two chambers through which sample or solvent can be passed. A separation membrane was positioned between two outer membranes (termed restriction membranes as their molecular mass cut-offs are usually smaller than the cut off of the separation membrane). When the cartridge was installed in the apparatus, the restriction membranes were located adjacent to an electrode. The cartridge is described in AU 738361, which description is incorporated herein by reference.

Description of membrane-based electrophoresis can be found in US 5039386 and US 5650055 in the name of Gradipore Limited, which description is incorporated herein by reference.

Polyacrylamide Gel Electrophoresis (PAGE)

Standard PAGE methods were employed as set out below.

Reagents: 10x SDS Glycine running buffer (Gradipore Limited, Australia), dilute using Milli-Q water to 1x for use; 1x SDS Glycine running buffer (29 g Trizma base, 144 g Glycine, 10 g SDS, make up in RO water to 1.0 L); 10x TBE II running buffer (Gradipore), dilute using Milli-Q water to 1x for use; 1x TBE II running buffer (10.8 g Trizma base, 5.5 g Boric acid, 0.75 g EDTA, make up in RO water to 1.0 L); 2x SDS sample buffer (4.0 ml, 10% (w/v) SDS electrophoresis grade, 2.0 ml Glycerol, 1.0 ml 0.1% (w/v) Bromophenol blue, 2.5 ml 0.5M Tris-HCl, pH 6.8, make up in RO water up to 10 ml); 2x Native sample buffer (10% (v/v) 10x TBE II, 20% (v/v)PEG 200, 0.1g/L Xylene cyanole, 0.1g/L Bromophenol blue, make up in RO water to 100%); Coomassie blue stain (Gradipure™, Gradipore Limited). Note: contains methanol 6% Acetic Acid solution for de-stain.

Molecular weight markers (Recommended to store at -20°C): SDS PAGE (e.g. Sigma wide range); Western Blotting (e.g. color/ rainbow markers).

SDS PAGE with non-reduced samples

To prepare the samples for running, 2x SDS sample buffer was added to sample at a 1: 1 ratio (usually 50 µL / 50 µL) in the microtiter plate wells or 1.5 ml tubes. The samples were incubated for 5 minutes at approximately 100°C. Gel cassettes were clipped onto the gel support with wells facing in, and placed in the tank. If only running one gel on a support, a blank cassette or plastic plate was clipped onto the other side of the support

Sufficient 1x SDS glycine running buffer was poured into the inner tank of the gel support to cover the sample wells. The outer tank was filled to a level approximately midway up the gel cassette. Using a transfer pipette, the sample wells were rinsed with the running buffer to remove air bubbles and to displace any storage buffer and residual polyacrylamide.

Wells were loaded with a minimum of 5 µl of marker and the prepared samples (maximum of 40 µl). After placing the lid on the tank and connecting leads to the power supply the gel was run at 150V for 90 minutes. The gels were removed from the tank as soon as possible after the completion of running, before staining or using for another procedure (e.g. Western blot).

Staining and De-staining of Gels

The gel cassette was opened to remove the gel which was placed into a container or sealable plastic bag. The gel was thoroughly rinsed with tap water, and drained from the container. Coomassie blue stain (approximately 100 ml Gradipure™,

Gradipore Limited, Australia)) was added and the container or bag sealed. Major bands were visible in 10 minutes but for maximum intensity, stained overnight. To de-stain the gel, the stain was drained off from the container.

The container and gel were rinsed with tap water to remove residual stain. 6% acetic acid (approximately 100 ml) was poured into the container and sealed. The de-stain was left for as long as it takes to achieve the desired level of de-staining (usually 12 hours). Once at the desired level, the acetic acid was drained and the gel rinsed with tap water.

Abbreviations

Acrylamide (AAm), N,N'-methylenebisacrylamide (BIS), poly(acrylamide) gel electrophoresis (PAGE), 2-hydroxyethyl acrylate (HEA), 2-hydroxyethyl methacrylate (HEMA), poly(ethylene glycol) acrylate (PEGA), poly(ethylene glycol) methacrylate (PEGMA), ethylene glycol diacrylate (EGDA), ethylene glycol dimethacrylate (EGDMA), poly(ethylene glycol) acrylate (PEGA), poly(ethylene glycol) methacrylate (PEGMA), poly(ethylene glycol) diacrylate (PEGDA), poly(ethylene glycol) dimethacrylate (PEGDMA), poly(ethylene glycol) PEG, and poly(propylene glycol) PPG.

EXPERIMENTAL

Hydrogels are polymers characterized by hydrophilicity and insolubility in water. Due to their potential biocompatibility, synthetic hydrogels are used extensively as prosthetic materials, soft contact lenses, membranes, controlled drug release materials, tissue engineering, and as separation matrixes in protein electrophoresis.

Hydrogels are predominantly formed by free radical co-polymerization (FRCP) of mono- and multi-unsaturated monomers. Despite several research attempts to prepare HEMA hydrogels in non-aqueous medium, hydrogels to be used in aqueous systems are on the whole synthesized in water. The choice of water as a solvent is convenient due to low cost, low toxicity, and the final usage of the gels in aqueous mediums.

In spite of these advantages, there are a number of limitations associated with the use of a pure aqueous solvent system. One such problem is the solubility of the monomer and the compatibility of the resultant polymer, which severely restricts both the choices and applicable concentration ranges of monomers. Hydrogels used as separation matrixes for bio-molecules are predominantly made from acrylamide and its derivatives. Despite its popularity, there are several limitations to acrylamide hydrogels

including the toxicity of the monomers, and the limited ranges of pore size obtained in such systems. Several research attempts have been made to produce macro-porous acrylamide hydrogels, but so far with limited success in separating large bio-molecules (> 2000 kDa) in preparative electrophoresis.

Amphiphilic polymer networks of 2-hydroxyethyl methacrylate (HEMA) have been studied extensively as materials for pharmaceutical and biomedical applications, including carriers for controlled drug delivery and materials for prosthetic devices. The mechanical strength of the hydrophobic backbone and the hydrophilicity of the hydroxyl and ester groups on the polymer side chains make polymers produced from HEMA excellent candidate for separation membranes.

Most existing HEMA systems are prepared in bulk, or with < 50% diluent. Owing to the hydrophobicity of the network, organic diluents are normally used. Although the properties of these hydrogels can be modified by crosslinking or by the use of different diluents, their swelling in water is thermodynamically limited to ~40% because of hydrophobic interaction within the network. As a result, such hydrogels are normally poor in mechanical strength (glassy and brittle), low in biocompatibility because of their low water content, and possess a limited pore size range. Such hydrogels have been predominantly used in applications that desire low water swelling, such as contact lenses and transport membranes for gases and ions.

The present inventors have found that by using a mixture of water and water-miscible entities as the polymerization solvent forming a hydro-organic system, HEMA hydrogels can be synthesized with low initial monomer content (5-40%). Using water-miscible entities such as oligomeric poly(ethylene glycol) and poly(propylene glycol), HEMA hydrogels that possess higher water swelling properties and larger pore sizes than existing hydrogels were successfully formed. Such hydrogels can be subsequently used as synthesized or after the water-miscible entities have been displaced with water.

It was also found that as the molecular weight of the water-miscible entities increases, the pore size of the hydrogels becomes dependent upon the properties of the entities, with the entities acting as a "porogen". In high molecular weight solvents, hydrogels synthesized in solutions of high molecular weight entities were observed to swell more than that of lower molecular weight.

Current membrane technologies for preparative electrophoresis of proteins are normally size-based or charge-based. The size-based separation is analogous with traditional ultra-filtration separations, with the transport processes dependent on the size of the pores in relationship to the size of the biomolecules. Consequently, this method is

limited by the pore sizes of the membrane, making the separation of large bio-molecular compounds from each other difficult.

Size exclusion chromatography (SEC) is another separation technique which separates solutes according to their size. In this chromatography, molecules are passed typically through a column packed with porous gel particles. The average diameter of the pores in the particles is of the same magnitude as that of the molecules, and hence, small molecules are able to diffuse into the gel, whereas larger molecules are prevented by their size from entering the pores. The larger molecules will have a shorter retention time and as a result, are separated and eluted before the smaller ones.

Parazeres (Chemical Engineering Science **1997**, 52, 953) was the first to suggest that ultra-filtration membranes with large pores (for convective flow), interconnected with smaller diffusive pores would have separation behaviour similar to that offered by a size-exclusion column. Such membranes would elute in decreasing order of size, and allows higher flow rates at lower pressures because of the physical form of the membrane (Figure 1). Using a thin layer of macroporous poly(vinyl alcohol) coated poly(styrene-co-divinylbenzene) as membrane in the electrophoresis of polymeric dyes, Budd et al (Price, A.D., Budd, P.M., Polymer Bulletin **2002**, 49, 33) subsequently showed that dyes with larger molecular weight exhibited higher mobility. In this technique, the macroporous membrane was synthesized by solvent casting a hydrophobic polymer in an organic solvent, followed by an additional step to increase the hydrophilicity of the surface.

By utilizing the observed "porogenic" effect of water-miscible entities in HEMA hydrogels, novel hydrogels and membranes with size exclusion properties for the separation of large bio-molecules have been developed. Two general types of pores exist in such membrane - large pores usually formed by the porogen, and small pores usually formed by the crosslinking of polymer chains. Such membranes can be made in a one-step process because the water-miscible entities are acting both as a porogen and a solvent for the amphiphilic polymer network. The membrane or medium formed has macropores and micropores which allow its use for SE applications.

The present inventors have developed a completely new SE type bio-macromolecular separation process.

Synthesis of novel hydrogels in hydro-organic solvents

The work started with the synthesis of novel HEMA hydrogels in solvents which consist of water and water miscible entities: Although monomeric HEMA is soluble in water, poly(HEMA) is immiscible with water over a large range of concentrations. The addition of water miscible entities can be used to alter the phase behaviour of the solvent-polymer system, hence increasing the miscible region for poly(HEMA).

Previous studies from our research group have shown that phase diagrams of linear polymers can provide a general guideline to the condition under which a clear hydrogel can be formed. We therefore propose a similar approach to this more complicated system in which the polymer is not soluble in one of the solvents. Linear poly(HEMA) was used to study the solvent effects in phase diagrams. As shown in Figure 2, the miscibility region for poly(HEMA) increases upon the addition of water miscible entities; in the region of the clear phase, a homogeneous solution was obtained, in the region of the opaque phase, phase separation occurs.

Various phase diagrams with different solvent composition and molecular weight of polymers can be produced, depending on the monomer used. The knowledge of these phase diagrams is very useful to transfer from a linear polymer to a polymeric network system. Using this principle, a new network formation path can be developed using a mixed solvent system, where monomer concentration from 10-40%M can be used to produce larger pore structure. Because of the use of such monomer concentration, the resultant hydrogel will provide good mechanical strength.

A range of water miscible entities with different molecular size have been investigated. Initially, ethylene glycol, propylene glycol and their polymeric analogues were used as the water miscible entity for hydrogel synthesis. The rate of polymerization, gel point, and monomer conversions then was studied. A number of techniques has been developed to monitor the polymerization process, including quantitative heat measurement, on-line Differential Scanning Calorimetry, and micro-particle detection.

The use of mixed solvents has significantly extended the range of monomers compared to that possible with only water as the solvent. It provides more options for the choice of the type of monomers that can be used in a specific application.

Size exclusion electrophoresis

Compared to column chromatography, which normally involve high pressure drops and compaction for soft gels at high flow rates, membrane chromatography has a lower pressure drop, high flow rate and high productivity as result of microporous / macroporous structures in relatively thin membranes.

As described above, protein separations under electrophoresis with a separation membrane are normally either size or charge based, which have limitations of its own such as the range of proteins can be separated. The present inventors have introduced the concept of protein separation under size exclusion chromatography principle using electrophoresis. By using this concept, protein can be separated in an opposite manner to conventional electrophoresis and some large bio-molecules, which are not able to be separated by existing systems, has been purified by this process.

The basic requirements for a SE separation are that the separation medium contains at least two types of pores: macropores and micropores. In chromatography, the large molecules will go though the big pores and travel fast while the smaller molecules will have interaction with small pores due to its compatible size with the micropores. Therefore in the separation of polymers by using size exclusion chromatography, polymer with largest molecular weight will elute out of a separating column first and the one with the smallest molecular weight will elute out last.

In the design of the SE hydrogel matrix systems, the present inventors have adopted the same principle. The solvent system used can act both as a porogen and a solvent to the amphiphilic monomer. The monomers used produce network structures with functional groups and these functional groups can interact with small proteins as these molecules enter the small pore structure. This is illustrated in Figure 3:

The hydrogels can be used in two different ways by utilizing the recently developed Gradiflow system to test the separation of the resultant membranes; one way is for the manufacture of membranes with a larger pore size or with improved functionality. The other is SE hydrogel electrophoresis.

Membranes with larger pore size can be tested in the following way: the membrane will be placed in the middle of a separation cartridge in a separation unit, as shown in Figure 4. The protein mixture to be separated will be placed in Upstream (Figure 4). When the charge is applied, the separation will begin and small proteins will travel to downstream through the membranes.

When SE type membrane is used, it is placed in the middle of a separation cartridge in a separation unit, as also shown in Figure 4. The protein mixture to be separated will be placed in Upstream (Figure 4). When the electric potential is applied, the separation will begin and large proteins will travel to downstream through the SE-type membranes. With the increase of time, small proteins may saturate the small pores of the separation membrane and the process needs to be pulsed to release the small proteins back to the upstream. This process can be carried out by removing separated proteins from downstream and reverse the potential supplied.

EXAMPLES

Example 1: Electrophoresis of polymeric dyes in 20%M 5%X HEMA/EGDMA synthesized in 50% aqueous PEG 6000 solution

Polymer-dye adducts of poly(vinyl alcohol)s (MW 72,000, 124,000 and 186,000) were prepared according to Budd (Price, A.D., Budd, P.M., Polymer Bulletin **2002**, 49, 33).

Aqueous solutions of PEG 6,000 (50%) were prepared. HEMA (3.84 g) and EGDMA (160.0 mg) were added to the above solutions (17 g) in disposable glass vials. The monomer solution was then degassed by argon purging for 5 min prior to the addition of the initiator system (0.2 mol% initiator per double bond) composed of freshly made up 10% (w/v) APS and 10% (v/v) TEMED. This mixture were then added to an upright mould comprising two glass plates (12 x 5 cm) separated by a rubber spacer (2 mm thickness) and the polymerization was allowed to proceed at room temperature overnight under an argon environment.

The above electrophoresis medium was immersed in water (1 kg) for 1 week during which the immersing solution (water) was exchanged on a daily basis. It was then saturated with pH 11 buffer (0.05M $NaHCO_3$, 0.01M NaOH) before use. Polymer-dye adducts samples were placed in sample wells and the electrophoresis was carried out at a field strength of 50 Vcm^{-1} for 1.5 hr using a horizontal electrophoresis system. Subsequent densitometric analysis of the hydrogel using a flatbed scanner showed the relative distance of migration of the polymer-dye adducts as a function of the molecular weight of the adducts, with the highest molar mass sample (MW 186,000) exhibiting the highest mobility.

Example 2: Electrophoresis separation analysis of 15%M 4%X HEMA/EGDMA membrane synthesized in 50% aqueous PEG 4000 solution

Aqueous solution of PEG 4000 (50%) was prepared. 15%M 4%X HEMA/EGDMA mixtures with the above PEG 4000 solution were polymerized into thin membranes with stacked Teric BL8 treated unwoven PET sheet as the supporting substrate.

Membrane-based electrophoresis analysis of a 15%M 4%X HEMA/EGDMA membrane synthesized in 50% aqueous PEG 4000 solution was performed using Bovine Haemoglobin (MW 64,000) and Lysozyme (14,400) as protein markers and NaMES/HMES buffer (pH 6). At pH 6, the infinite dilution mobility of Haemoglobin and Lysozyme are 0.5 and 0.6 10^{-4} $cm^2V^{-1}s^{-1}$ respectively (Ho, A. *Protein electrophoresis through porous membranes*, PhD Thesis, Department of Chemical Engineering, The University of Melbourne, Australia (2000)).

SDS-PAGE analysis of samples taken from the two streams of the unit during the experiment showed that Haemoglobin was the first protein to be separated from stream 1 to stream 2.

SUMMARY

Examples 1 and 2 demonstrate that by careful selection of the types and concentrations of the water-miscible entities, hydrogels with size exclusion properties can be obtained. Example 1 shows the separation of poly(vinyl alcohol) molecules, Example 2 shows the separation of two different sized proteins that have similar electrophoretic mobilities.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this eighteenth day of December 2002

The University of Melbourne

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PATENT & TRADE MARKS ATTORNEYS

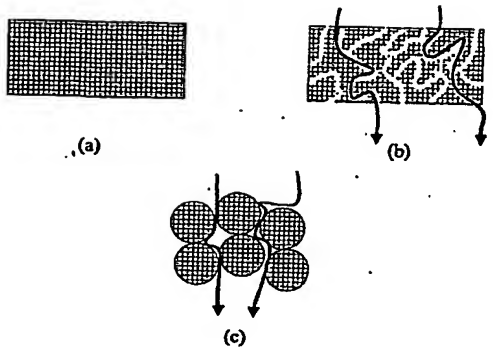

Figure 1

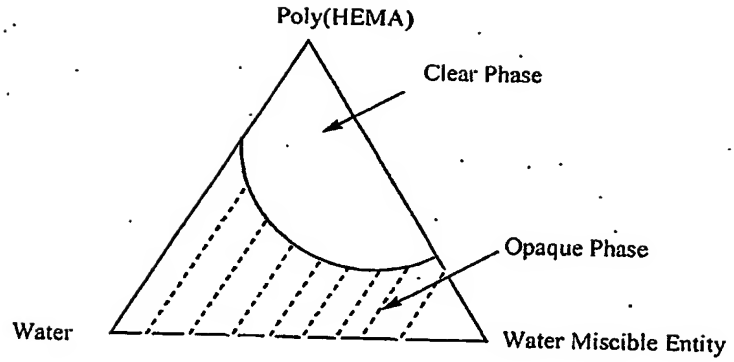

Figure 2

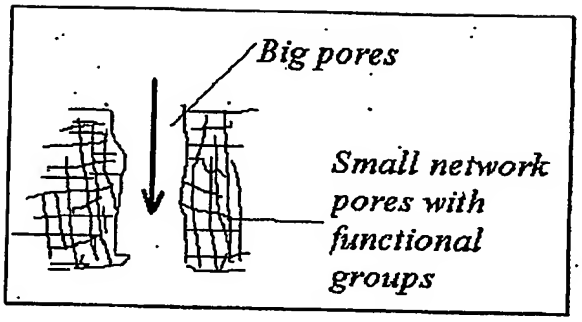

Figure 3

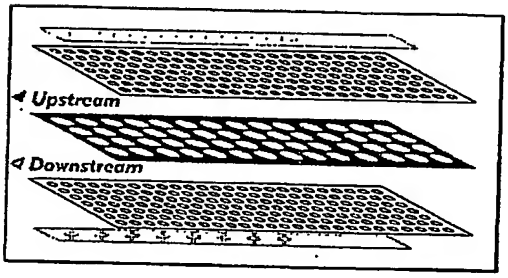

Figure 4